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Endocrine and Ultrasonographic Characterization of a Successful Pregnancy in a Sumatran Rhinoceros (*Dicerorhinus sumatrensis*) Supplemented With a Synthetic Progestin

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A Sumatran rhinoceros with a history of early pregnancy loss was supplemented with a synthetic progestin, altrenogest (Regu-Mate®), and delivered a healthy, full-term calf 475 days after mating. Serum hormone concentrations were measured throughout gestation, and ultrasonography was used to monitor embryo/fetal growth and viability. The embryonic vesicle growth curve was characterized by three phases: rapid expansion, plateau, and a final rapid expansion, and was similar to that in the domestic horse. Fetal sex was determined by ultrasound on day 73 of gestation. After day 80 of gestation, transabdominal examinations were more useful than rectal examinations for imaging the fetus. Serum progesterone concentrations remained at luteal levels (1.5 ± 0.5 ng/ml) for the first 2 months of pregnancy, and then they gradually increased. However, progesterone decreased almost to luteal levels during the fifth month before it increased again, and eventually reached peak concentrations (13.3 ± 1.9 ng/ml) shortly before parturition. Relaxin concentrations remained basal (≤ 0.5 ng/ml) for the first half of the pregnancy, increased to 2.7 ± 1.2 ng/ml and stabilized until 2 weeks before parturition, when relaxin spiked to unusually high concentrations (800–1300 ng/ml). Prolactin concentrations were at baseline

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(7.2 ± 1.7 ng/ml) throughout most of the gestation, but rose markedly 2 weeks before parturition, reaching concentrations as high as 75 ng/ml. Attempts to measure serum estrogen concentrations were unsuccessful. These data represent the first attempt to characterize pregnancy in the critically endangered Sumatran rhinoceros, a species that heretofore had not successfully reproduced in captivity for 112 years. Zoo Biol 23:219–238, 2004.

Key words: prolactin; relaxin; progesterone; embryo; fetus; parturition

INTRODUCTION

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is one of the most endangered mammals on earth, and it is estimated that fewer than 300 currently exist in the wild [Khan et al., 1999]. Because this species is on the brink of extinction, largely due to poaching and, to a lesser extent, habitat destruction, a formal captive breeding program was established in the mid-1980s. Forty animals were brought into zoos and captive breeding centers in the United States, Europe, Malaysia, and Indonesia [Foose and Van Strien, 1997; Khan et al., 1999]. Unfortunately, as a result of health problems and difficulties in breeding this species, the captive population had dwindled to less than half the original number by the late 1990s [Khan et al., 1999].

Breeding Sumatran rhinoceroses has proven challenging because the animals can become very aggressive when they are paired and the female is not receptive to the male. Because neither the male nor the female exhibit reliable behavioral signs indicating the female is in estrus, these animals were often introduced when the female was not receptive, and subsequent aggression sometimes resulted in serious injuries [Khan et al., 1999]. More recently, studies involving endocrine monitoring and serial ultrasound examinations have increased our understanding of this species' unusual reproductive physiology. The discovery that the Sumatran rhinoceros appears to be an induced ovulator with unique patterns of progesterone secretion was a key factor in the development of a reliable method to determine when males and females should be paired for mating [Roth et al., 2001]. However, although a pair of Sumatran rhinoceroses were repeatedly mated and multiple pregnancies resulted, recurring early pregnancy loss hindered the ultimate goal of producing offspring in this species [Roth et al., 2001].

Early pregnancy loss has been reported in numerous domestic and nondomestic species. The closest domestic relative of the rhinoceros, the horse (*Equus caballus*), is known for its relatively high rate of early pregnancy loss. On average, approximately 10% of mares lose their pregnancies in the first 50 days of gestation, and much higher losses are reported for subfertile mares [Ball, 1988; Ginther, 1995]. The prevalence of spontaneous abortion in rhinoceroses is unknown, but it has also been documented in captive black [Berkeley et al., 1997] and white [Radcliffe et al., 1997] rhinoceroses, and a recent report suggested that it even occurs in wild black rhinoceroses [Garnier et al., 2002]. Early pregnancy loss in the mare is most often associated with endometrial inflammation and fluid accumulation within the uterus, and is more prevalent in older mares [Carnevale and Ginther, 1992]. Low serum progesterone levels also have been associated with early pregnancy loss in mares [Bergfeldt et al., 1992]. Although luteal insufficiency has not been proven to be a primary cause of pregnancy loss [Ginther, 1995], the oral progesterone supplement

altrenogest is commonly prescribed in suspected cases and appears to be the only form of progesterone associated with sustaining pregnancy [McKinnon et al., 2000]. Furthermore, altrenogest is apparently safe [Shideler et al., 1983; Squires et al., 1983] and does not effect the fertility of resulting offspring [Naden et al., 1990]. Therefore, it has also been administered to a few exotic species in which pregnancy loss due to low progesterone is suspected. Full-term pregnancies resulted when an okapi (*Okapia johnstoni* [Schwarzenberger et al., 1999]) and a black rhinoceros (*Diceros bicornis* [Berkeley et al., 1997]), both of which had a history of early abortion, were supplemented with altrenogest. No negative side effects were reported.

The last record of a Sumatran rhino calf produced by a pair of captive animals dates back to 1889, in Calcutta, India [Rookmaaker, 1998]. Although wild rhinoceroses have been studied [Van Strien, 1985], the secretive nature of this forest-dwelling species has severely limited the collection of data regarding reproductive characteristics. Therefore, information about pregnancy in the Sumatran rhino is almost nonexistent, and what little there is, is questionable. For example, Bartlett [1873] described a 7-month gestation period for this species. This information has been reported in many encyclopedias [Macdonald, 1987], even though three other studied rhino species are known to have a 14–17-month gestation. Although the endocrine database for pregnant African black and white rhinoceroses has grown significantly in the past decade [Schwarzenberger et al., 1993, 1998; Berkeley et al., 1997; Radcliffe et al., 1997; Patton et al., 1999; Schwarzenberger et al., 2000; Brown et al., 2001], data on endocrine patterns in the Sumatran rhinoceros are scarce [Heistermann et al., 1998; Roth et al., 2001], and no hormonal profiles for a successful Sumatran rhino pregnancy have been reported. Studies detailing behavioral or morphological changes associated with pregnancy or pending parturition in this species are also lacking. This paucity of information is not surprising considering that only four Sumatran rhino calves have been born in captivity, three were from females that were already pregnant when they were captured in the wild [Rookmaaker, 1998], and all but one were born over a century ago.

The ultimate goal of this study was to overcome the problem of recurrent early pregnancy loss in a Sumatran rhinoceros and produce a viable calf. Supplementation with altrenogest was tested in an effort to achieve this objective. A secondary goal was to obtain a comprehensive (albeit preliminary) set of data regarding pregnancy in this rhino species. The specific objectives were to 1) characterize early embryo development and fetal dynamics throughout gestation using ultrasonography; 2) evaluate serum progesterone, estrogen, prolactin, and relaxin concentrations; and 3) record behavioral and morphological changes in the female rhinoceros associated with the prepartum period and imminent parturition.

MATERIALS AND METHODS

Animal Care and Progesterone Supplementation

A pair of Sumatran rhinoceroses, on loan to the United States from the Indonesian government, were maintained at the Cincinnati Zoo & Botanical Garden. The female was captured as a 1-year-old on the island of Sumatra and was raised at the Los Angeles Zoo. She was 11–12 years old during this study. The male

was captured in Sumatra as an adult and was estimated to be >20 years old. From September 1997 to May 2000, the female became pregnant five times and lost each pregnancy within the first 3 months of gestation [Roth et al., 2001].

The rhinoceroses were provided a diet that consisted primarily of fresh ficus (30–50 kg per day) supplemented with one or two flakes of hay (40% alfalfa and 60% orchard grass), 1.8 kg of pelleted feed (ADF 16; Mazuri, St. Louis, MO) and a variety of fresh fruits (e.g., apples and bananas) and vegetables (e.g., sweet potatoes and carrots). The rhinos had unlimited access to fresh water and iodized salt blocks. Each morning, they received a 6-ml vitamin E supplement (EmcelleTocopherol, 500 U/ml; Stuart Products Inc., Bedford, TX) fed in a banana. Daily weights were obtained by walking the animals onto a floor scale.

The rhinoceroses were maintained separately in adjacent enclosures except when the female was thought to be in estrus, based on serum progesterone values and ovarian follicle size [Roth et al., 2001]. On those days the animals were paired for mating, which usually took place within an hour or two following their introduction. After they mated, the animals were immediately separated and were then housed individually. Each rhinoceros had access to a stall in a heated barn. During the summer months, they spent several hours each day outside on public display, with access to a pool. During the winter months, outdoor access was limited and depended on the weather. The barn was maintained at 21°C and the animals were exposed to artificial lighting from 0700 to 1800 hr until 6 weeks prior to parturition, when the female rhinoceros was also exposed to dim lighting from 1800 to 0700 hr.

Altrenogest (Regu-Mate® Intervet Inc., Millsboro, DE), a synthetic progestin, was administered daily following ultrasonographic confirmation that the female was pregnant. This was the sixth pregnancy diagnosed in this female over a 3-year period. Using the dosage prescribed for horses, the 799-kg rhinoceros ingested 16 ml of Regu-Mate® (0.044 mg/kg body weight) each morning. To ensure that the full dosage was received, the animal-care staff slowly expelled 16 ml of Regu-Mate® into a stack of six to eight pieces of white bread. As the bread soaked up the liquid progesterone, it was tossed into the open mouth of the female rhinoceros, which then proceeded to promptly chew and swallow the bread (and thus the entire hormone dosage). Altrenogest supplementation was initiated 18 days after mating and continued until day 465 of pregnancy. Starting on day 450, the staff weaned the female from the Regu-Mate® by slowly reducing the daily dosage 1.0 ml each day until she was no longer receiving any supplement (day 465).

Ultrasonography

The female rhinoceros previously had been conditioned to allow frequent ultrasound examinations without the use of sedatives or anesthesia [Roth et al., 2001]. An Aloka 500V ultrasound machine (Aloka, Wallingford, CT) connected to a video cassette recorder was used for all examinations. A Sony thermal printer (Aloka) was used to print the images. All rectal examinations were conducted using a 5-MHz linear array probe, and transabdominal examinations were conducted using a 3.5-MHz convex probe. No modifications of the probes were necessary.

Rectal ultrasound examinations were performed prior to mating to monitor ovarian follicular development. At 50 hr postmating, an ultrasound examination was performed to confirm ovulation, and 17 days after mating another one was conducted to detect pregnancy. From day 21–84 of pregnancy, intensive ultrasound

monitoring (every 9–11 days) was conducted because the female had lost five previous pregnancies during this early stage of gestation. From day 84–257 of pregnancy, examination frequency was decreased to just twice each month, and then to just monthly exams from day 257–459. Transabdominal examinations were initiated on day 117 of pregnancy and were carried out in conjunction with rectal exams through day 459 of pregnancy.

Early-pregnancy data collected via ultrasound during this pregnancy, the first five failed pregnancies, and a pregnancy that was established while this manuscript was being prepared included measurements of early embryonic vesicle diameter, embryo proper formation and orientation within the vesicle, early fetal crown–rump length (CRL), and detection of a heartbeat. In the successful pregnancy, we also monitored additional changes to the reproductive tract, such as cervical orientation, placental formation, and fetal positioning. The examinations always included a thorough scan of the cervix, evaluation of the allantoic fluid, and frequent assessments of placental development in the nongravid horn. Fetal imaging was always attempted, but was not always successful. Mammary gland palpation was conducted concurrently with ultrasound examinations during the last 2 months of gestation to detect early swelling of the tissue that might suggest pending parturition.

Hormonal Analyses

Blood was collected throughout gestation for hormone analyses. The female previously had been conditioned to allow blood to be collected from her ears. A tourniquet was placed at the base of the ear and a 23-gauge butterfly catheter attached to a 6-ml syringe was used to collect the sample. Blood was collected weekly for the first 3 months of gestation, and then every 2 weeks until day 450 of pregnancy. At day 450, when the supplemental progesterone dosage was decreased, and until after parturition, blood was collected every 3 or 4 days. The samples were centrifuged ($1300 \times g$) and then stored in 1.0-ml aliquots at $-80^{\circ}C$ until they were thawed for analysis.

Progesterone enzyme immunoassay (EIA)

All of the serum samples collected throughout gestation were analyzed for progesterone concentrations. Serum progesterone was extracted and the concentrations were determined by EIA following the protocol described by Munro and Stabenfeldt [1984] and using a monoclonal antibody produced against 4-pregnen-11-ol-3,20-dione hemisuccinate: BSA (provided by J. Roser, University of California–Davis). This assay was previously validated for Sumatran rhinoceroses [Roth et al., 2001]. Assay sensitivity was 0.039 ng/well, and an extraction efficiency of 85% was used to calculate actual serum concentrations.

Relaxin radioimmunoassay (RIA)

A subset of serum samples ($n = 26$) from 1 day before mating to 4 days postpartum were analyzed for relaxin. The R6 homologous porcine relaxin RIA was conducted as previously described [O'Byrne and Steinetz, 1976], except that the incubations were shortened to 24 hr, and the antibody-bound labeled tracer was precipitated with a mixture of cold goat anti-rabbit IgG and polyethylene glycol, exactly as described previously for the canine relaxin RIA [Steinetz et al., 1996]. Purified porcine relaxin was used as the standard, and ^{125}I-labeled monotyrosyl

porcine relaxin was used as the radioligand. Rabbit anti-porcine relaxin antiserum R6 was used at a concentration of 1:20,000. All standards (1–64 ng/tube) and unknown samples were run in triplicate. Samples of 10, 50, or 150 μl were evaluated depending on the concentrations of relaxin present in the sample. The assay was validated for the species by confirming that dilution curves of pooled Sumatran rhino pregnancy sera and milk were parallel to that of porcine relaxin standards in assay buffer. Concentrations of relaxin in the rhino samples were calculated as ng/ml porcine relaxin equivalents. Coefficients of variation ranged from 10% to 18%, and the minimal detectable concentration was 1 ng/tube. The R6 antiporcine relaxin antiserum has been found to cross-react specifically with the receptor-binding domain of the B-chain (especially the arginine residues at positions B13 and B17 [Schwabe and Büllesbach, 1994]), and thus it recognizes relaxins from all species thus far sequenced.

Prolactin RIA

A subset of serum samples ($n = 30$) from 4 days prior to mating to 120 days postpartum were analyzed for prolactin by Dr. A.F. Parlow (National Hormone and Peptide Program, Harbor-University of California, Los Angeles Medical Center, Torrence, CA). Serum prolactin concentrations were determined using an equine prolactin RIA (RIA #26). The assay was validated for the Sumatran rhinoceros by confirming parallelism of serially diluted pooled rhino serum with similarly diluted equine prolactin (ePRL AFP7730B; National Hormone and Peptide Program, UCLA, Medical Center). The antibody used was a rat anti-ePRL antibody (AFP361687; National Hormone and Peptide Program, UCLA Medical Center) at a final dilution of 1:50,000.

Estrogen EIA

Serum samples were evaluated by EIA for estrone glucuronide and estradiol following methods previously described by Stabenfeldt et al. [1991]. The estrone glucuronide and estradiol antibodies (#R522 and #R4972, respectively; from C. Munro, University of California–Davis) were diluted 1:5,000 and 1:10,000, respectively. The HRP-conjugated estrone glucuronide and estradiol were used at final dilutions of 1:25,000 and 1:50,000, respectively. The sensitivity range of both assays was 3.9–250 pg/well. A pooled serum sample composed of both pregnant and nonpregnant samples from the female Sumatran rhinoceros was tested both raw and following petroleum ether extraction per the standard method used in the progesterone EIA [Munro and Stabenfeldt, 1984]. The samples, run in duplicate, were added to the wells neat and serially diluted from 1:2 to 1:128 to test for parallelism with the standard curve. Additionally, several samples from early, mid, and late gestation, and postpartum were concentrated by extracting 300 μl in petroleum ether followed by reconstitution in just 120 μl of EIA buffer. A 20-μl aliquot of the concentrated samples, representing 50 μl of extracted serum, was added to each well in duplicate.

Behavioral Observations

A behavioral ethogram was developed to monitor the female rhino's behavior starting several weeks prior to anticipated parturition to determine the normal frequency patterns of each behavior for this rhinoceros so that changes associated

with pending parturition could be detected. Dim lighting was installed, and two remote surveillance cameras were strategically placed in each of the two stalls occupied by the female rhinoceros overnight. Volunteers remotely monitored the animal by continuously recording data in 4-hr blocks for 16 consecutive hours starting at 1600 and ending at 0800 hr. The 17 recorded behaviors were defecating, drinking, eating, hind-leg kicking, horn rubbing, locomoting, lying laterally, lying sternally, pawing, playing with toys, rubbing, standing idle, standing up, straining, urinating, producing vaginal discharge, and vocalizing. Each time a behavior was exhibited, it was accounted for and the tick marks were summed up for each behavior within each 4-hr shift.

Statistical Analysis

The behavioral data were analyzed by one-way analysis of variance (ANOVA), and means within each data set were compared using Fisher's least significance difference (FLSD) test (Statview 5.0.1 MacIntosh statistical software package). The initial analysis was conducted by testing "week" as the main effect. Based on these results, analyses of behaviors in weeks 1–6 were conducted to determine the effect of "shift." Finally, within week 7, the effect of "day" was tested.

RESULTS

Weight Data and Udder Development

The female rhino's weight averaged 799 kg during the month in which she conceived, which is considered higher than optimal for this animal. The month prior to parturition, she weighed 807 kg, which indicates a total weight gain of just 8 kg during the 16-month gestation. Because the female was heavy at conception, the keeper staff was careful to not substantially increase her feed ration during pregnancy. Therefore, weight gain was minimal, and the female was in good body condition at the time of parturition.

Udder development was first indicated 2 weeks prior to parturition by a slight swelling of the mammary gland that was noticeable only upon palpation. Teat enlargement and mammary gland swelling continued to progress daily until parturition. About 1 week prior to parturition, the teats and mammary gland became visible between the hind legs of the female as she walked, but the udder never became engorged. Another morphological indicator of pending parturition was the relaxation of the vulva. During the 2 weeks prior to parturition, the vulva became relaxed, extended, and edematous in appearance. These changes in soft tissue tone were especially noticeable when the female was lying down.

Ultrasonography

The female rhino's ovaries contained two preovulatory follicles measuring 21 mm (right ovary) and 22 mm (left ovary) 24 hr before mating took place. Ovulation of both of these follicles was confirmed 50 hr after mating. Pregnancy was confirmed 17 days after mating by the presence of two embryonic vesicles measuring 18 mm and 10 mm in diameter. The smaller of the two vesicles underwent atresia (day 21, 9 mm; day 31, <5 mm) until it was no longer visible (day 41). The larger vesicle

continued to develop, and data were collected by a series of 29 ultrasound examinations over the 16-month gestation.

Embryonic vesicle diameter and fetal CRL data were collected until days 63 and 80, respectively, when the size exceeded the machine's capacity to obtain accurate measurements. Data from the sixth pregnancy were combined with previous data from the first three unsuccessful pregnancies [Roth et al., 2001] and data from a pregnancy established during the preparation of this manuscript to produce graphs depicting embryonic vesicle growth rate (Fig. 1a) and fetal CRL (Fig. 1b) in the Sumatran rhino. These data were combined only after the data from each pregnancy were plotted individually, and compared with similar data from the successful pregnancy to ensure there were no significant differences in data points or patterns. In the first three pregnancies, data obtained on the day the examination revealed that early embryo loss had occurred (as assessed by the absence of a vesicle or the presence of a shrunken vesicle) were not included.

The growth pattern of the Sumatran rhino embryonic vesicle was characterized by an early rapid expansion phase from day 14 until day 21, during which the vesicle increased in diameter approximately 2.5 mm per day. The vesicle then stopped expanding, and for about 7 days (days 21–27) it became less spherical. By day 28, expansion resumed and the vesicle increased approximately 3.0 mm per day in diameter until day 63, after which it was too large to measure accurately. The plateau phase coincided with cessation of embryonic migration through the uterus, formation of the embryo proper, and rotation of the embryo from a ventral to a dorsal position within the vesicle. The second rapid growth phase of the vesicle was associated with the descension of the embryo to the center of the vesicle from its dorsal location (day 41) and continued fetal growth. The CRL (Fig. 1b) became measurable at about 26 days, but it changed little until day 41. After day 41 the fetus grew rapidly, increasing in length from 14 mm to 80 mm in approximately 40 days.

During the ultrasound examination on day 63, it became evident that the placenta was invading the nongravid left horn. The embryonic vesicle had undergone fixation between days 21 and 31 and was located in the caudal section of the right uterine horn. By day 63, allantoic fluid surrounded by placental tissue could be visualized in the caudal section of the left horn (Fig. 2a). Placental invasion of the nongravid horn progressed over time, and the horn expanded as it filled with allantoic fluid (Fig. 2b). Through much of the pregnancy, the placenta and allantoic fluid in the nongravid horn could be evaluated during rectal ultrasound examinations.

Video footage obtained during an ultrasound examination on day 73 provided the imaging required for fetal sex determination. Based on a genital tubercle located closer to the umbilicus and more distant from the tail (Fig. 2c), the fetus was diagnosed as male by Dr. Richard D. Holder (Hagyard-Davidson-McGee Associates, Lexington, KY).

After day 80, the fetus descended over the pelvic brim and was no longer visible by rectal examination. Rectal exams continued to provide information regarding the opacity of the allantoic fluid and placental development. Transabdominal examinations in the area of the soft mammary tissue provided occasional imaging of the fetus until day 223 (Fig. 2d), after which the fetus moved cranially and could no longer be reliably seen. It was also at this time that the orientation of the cervix shifted from a

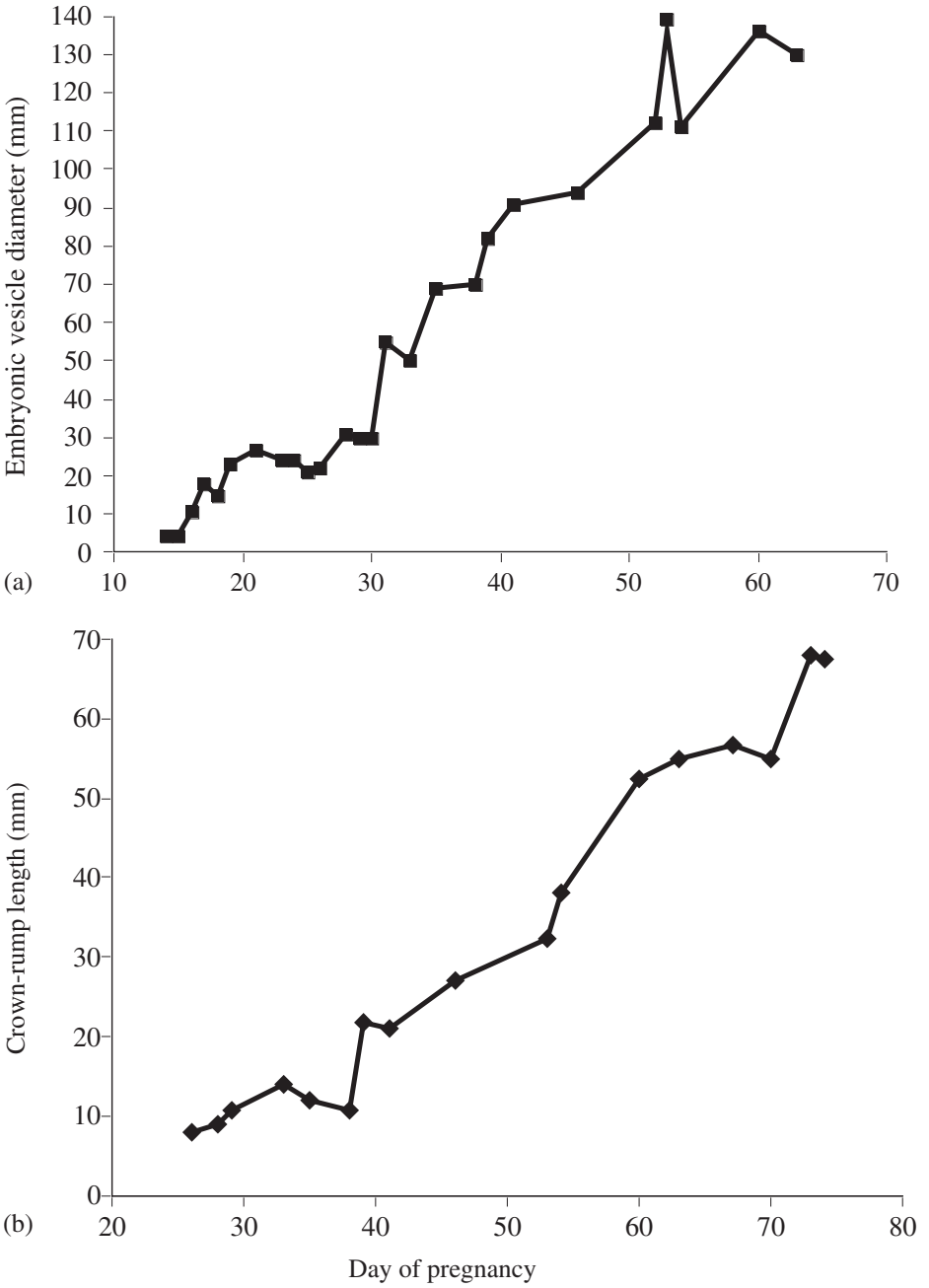
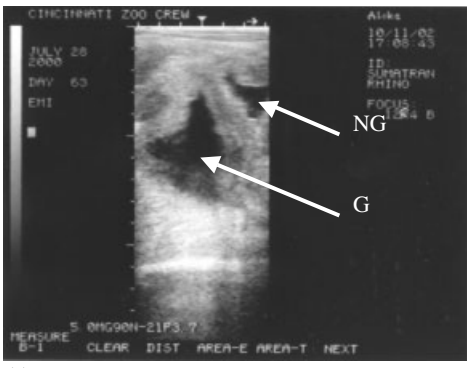
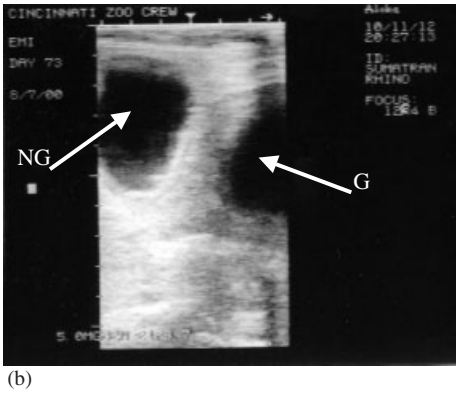


Fig. 1. Sumatran rhino (**a**) embryonic vesicle and (**b**) fetal growth rate during early pregnancy. Day 0 is the day of mating. For each data point, n = 1–4.

horizontal to a slightly vertical position (Fig. 2e), probably because of the weight and pull of the growing fetus. During the last 2 months of gestation, the fetus once again became visible during rectal examinations.



(a)



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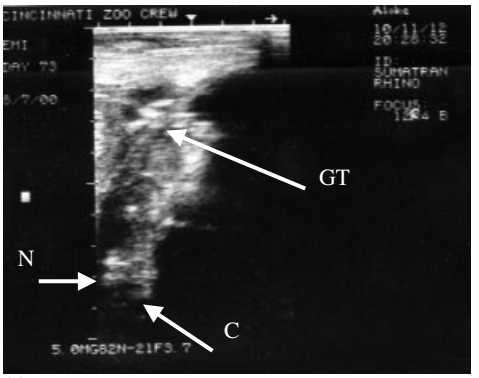

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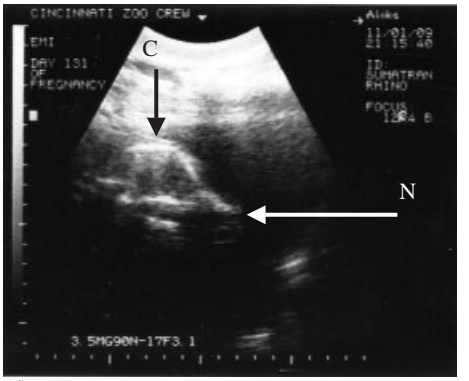
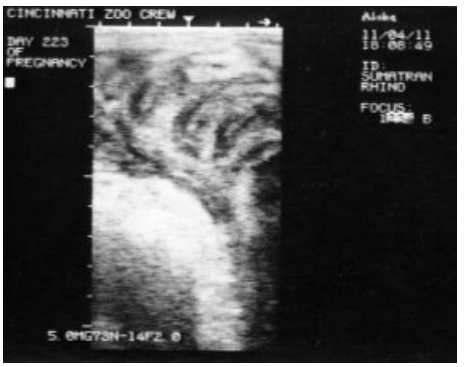


(d)



(e)

Fig. 2. Ultrasound images acquired during pregnancy in a Sumatran rhinoceros. **a:** Placental invasion of the nongravid (NG) horn was apparent on day 63 of pregnancy. **b:** By day 73 of pregnancy, both gravid (G) and NG horns were enlarged and filled with allantoic fluid. **c:** Fetal sex was determined on day 73 based on the location of the genital tubercle (GT) identified in an image of the fetus suspended vertically by the umbilicus with the fetal head ventrally oriented (crown (C) and nose (N)). **d:** Profile of the fetal head observed during a transabdominal ultrasound examination on day 131 of pregnancy. **e:** A shift in cervical orientation from horizontal to vertically sloping occurred by mid gestation.

Hormonal Analyses

Progesterone concentrations (Fig. 3a) rose from baseline levels (<0.1 ng/ml) on the day of mating to peak luteal levels 10 days later, and remained there for the first 2 months of gestation (mean ± SD; 1.52 ± 0.55 ng/ml). Progesterone then gradually increased during the third month to reach concentrations >4.0 ng/ml

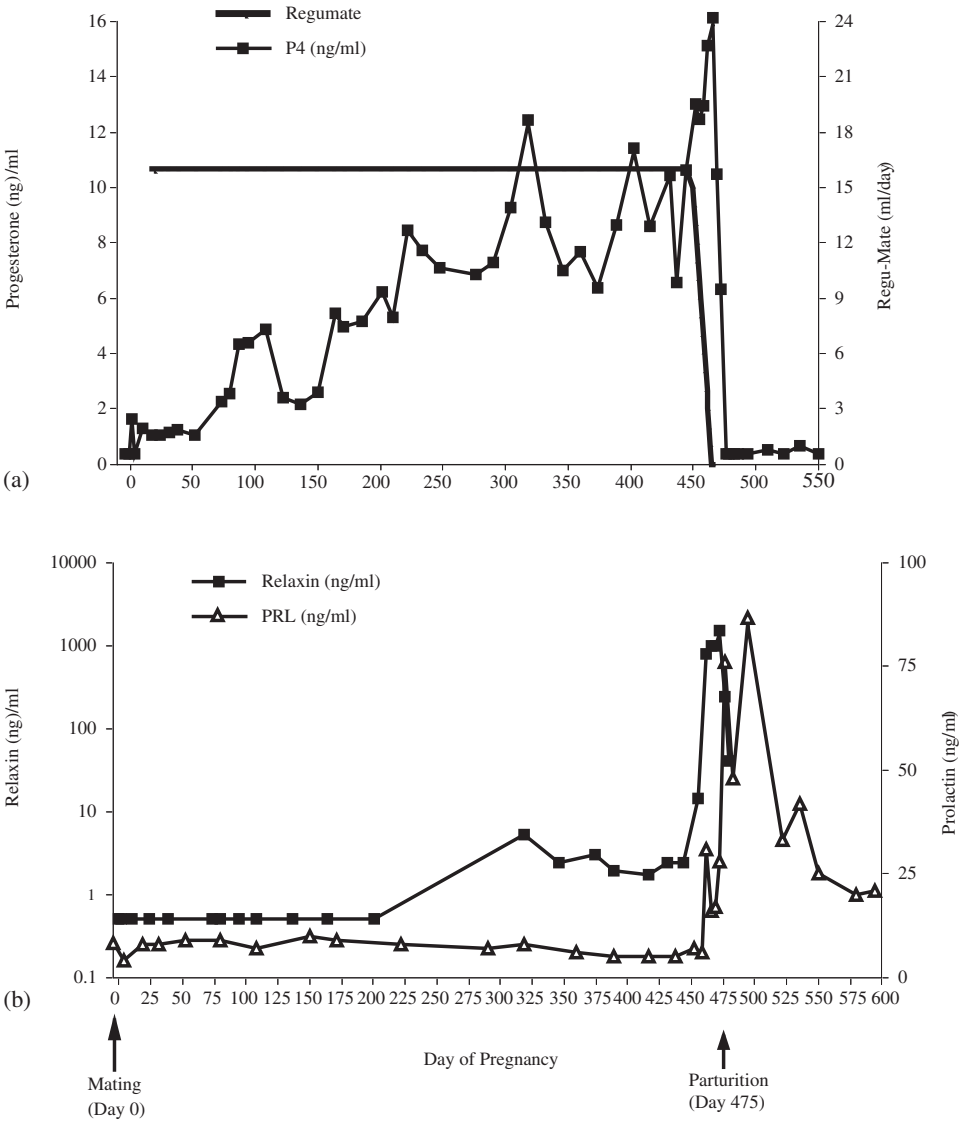


Fig. 3. Endocrine profiles characterizing a successful pregnancy in a female Sumatran rhinoceros supplemented with Regu-Mate®. **a:** Endogenous progesterone concentrations (■) and the daily dosage of Regu-Mate® (—) are reported from 4 days before mating until 75 days postpartum. **b:** Relaxin (■) and prolactin (△) concentrations were evaluated from 4 days before mating until 4 and 125 days postpartum, respectively.

during the fourth month. However, during the fifth month of gestation, progesterone levels decreased to almost 2.0 ng/ml and remained there for 4 weeks before they rose back up to 4.0 ng/ml and higher. From 7–15 months of gestation, progesterone levels averaged 8.54 ± 1.75 ng/ml (range, 6.37–12.32 ng/ml). Between days 450 and 465 (the same interval during which the Regu-Mate® dosage was decreased by 1 ml per day) progesterone levels appeared to increase further, rising to 13.31 ± 1.95 ng/ml (range = 10.48–15.89 ng/ml). Interestingly, during the week just prior to parturition, progesterone levels declined from 15.89 ng/ml on day 466 to 10.48 ng/ml on day 469, and 6.35 ng/ml on day 472, just 3 days before the female gave birth. By 24 hr postpartum, progesterone concentrations had returned to baseline (<0.1 ng/ml).

Relaxin concentrations (Fig. 3b) were basal (<1 ng/ml) for the first 7 months of gestation. By the 10th month, relaxin concentrations had increased substantially and averaged 2.71 ± 1.17 ng/ml until they spiked to ≥ 800 ng/ml 2 weeks before parturition. Due to a shortage in stored serum collected during months 8 and 9, relaxin concentrations could not be evaluated during that interval. Therefore, the timing of the first rise in relaxin cannot be more accurately determined. Relaxin concentrations decreased precipitously following parturition on day 475, with concentrations of 1,388 ng/ml on day 472, 241 ng/ml on day 476 (one day after parturition), and 41 ng/ml on day 479.

Prolactin concentrations (Fig. 3b) remained constant throughout the first 15.5 months of the pregnancy (7.17 ± 1.69 ng/ml) before they increased substantially between day 458 (6 ng/ml) and day 462 (31 ng/ml). Prolactin remained elevated (33.6 ± 24.6 ng/ml) throughout the last 2 weeks of gestation, but varied significantly among samples (range = 16–76 ng/ml). Prolactin concentrations were still elevated (21 ng/ml) 125 days postpartum.

Attempts to measure serum estrogen concentrations using the estradiol and estrone glucuronide EIAs failed to detect any measurable estrogen in either raw serum or extracted samples that were tested neat or diluted. All values for all dilutions (neat to 1:128) of both sample types were below the lowest standard (3.9 pg/well). Therefore, no parallelism was observed when values for serially diluted pooled serum samples were compared to the standard. In fact, assay values for all dilutions of both sample types were constant at 2–3 pg/well. Similarly, estradiol in the concentrated samples was not measurable. All concentrated samples produced results at or below the minimum sensitivity of the assay, suggesting estradiol concentrations in serum were consistently <0.078 ng/ml.

Animal Behavior

Because the frequency of each behavior rarely differed during the first 6 weeks, but the frequency of most behaviors differed in week 7 (the week of parturition), data from weeks 1–6 were used to determine the normal frequency patterns of each behavior for this rhinoceros. A summary of the behavioral data collected during weeks 1–6 of the observational study confirmed anticipated patterns of rhino activity: the active behaviors more frequently exhibited during the 1600–2000 and 0400–0800 hr shifts, while the inactive behaviors were more frequently recorded during the 2000–2400 and 2400–0400 hr shifts. The incidence of several behaviors, including urinating, pawing, vocalizing, and horn-rubbing increased ($P<0.01$) in the week of parturition (week 7) compared to the first 6 weeks of observations. When data for each day of week 7 were compared, it became clear that the shift in behavior

frequency occurred about 24 hr before parturition (on the Wednesday before and the Thursday of parturition; Fig. 4). The most obvious behavioral change was in the pattern of urination. Whereas during the first 6 weeks of the study, the female urinated no more than twice in each 4-hr shift, 12 hr prior to parturition she urinated more than 20 times each shift. Furthermore, the style of urination changed from a typical downward steady stream flow to distinct upward spraying squirts.

DISCUSSION

This work provides the first physiological data characterizing a successful pregnancy in the critically endangered Sumatran rhinoceros. In addition to providing data on the commonly studied steroid hormone progesterone, this report also includes profiles throughout gestation for prolactin and relaxin, two hormones for which no data have been reported in any rhino species. Because rhino endocrinology has been studied quite successfully by noninvasive fecal and urine

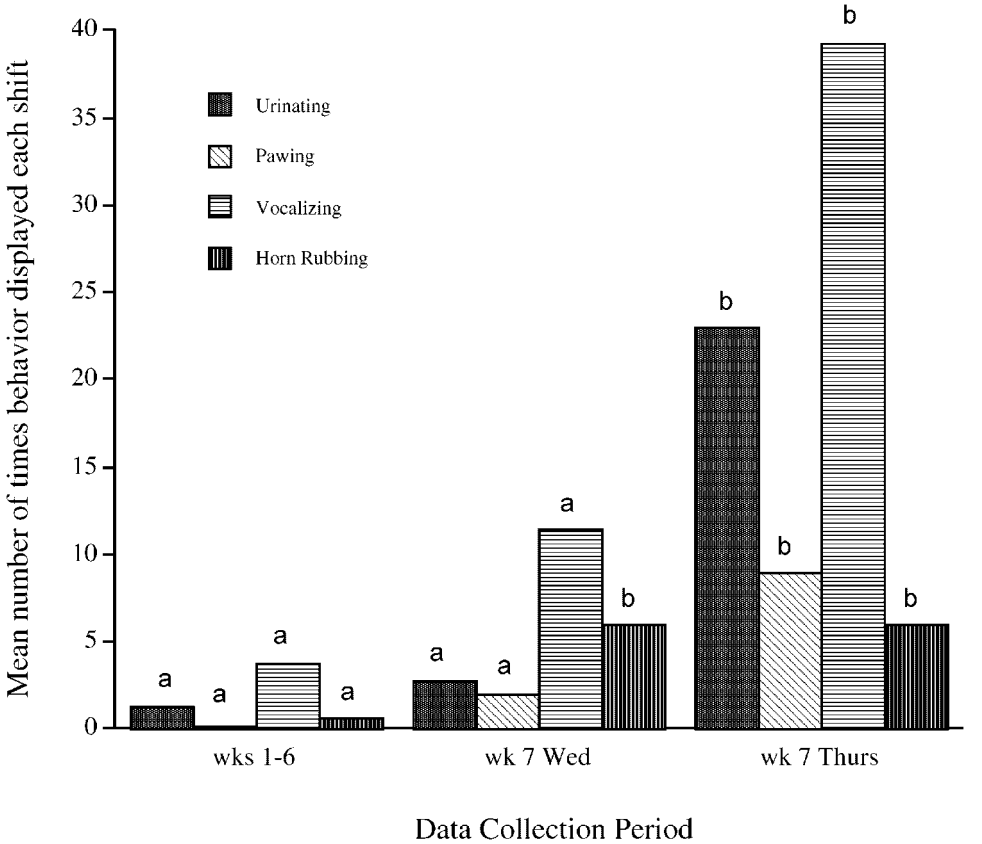


Fig. 4. Mean frequency per 4-hr shift of four behaviors: (▦) urinating, (▧) pawing, (▭) vocalizing, and (▥) horn-rubbing exhibited by a pregnant Sumatran rhinoceros during the 6 weeks prior to parturition, and the day before (Wednesday) and day of (Thursday) parturition (week 7). Different superscripts designate differences ($P < 0.05$) in the frequency of this behavior between data collection periods.

hormone metabolite monitoring, a great deal is known about hormone metabolite profiles in pregnant African and Indian rhinoceroses [Kasman et al., 1986; Hodges and Green, 1989; Schwarzenberger et al., 1993, 1998, 2000; Berkeley et al., 1997; Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001]. In contrast, serum hormone data is scarce in the literature and has only been reported for two rhino species [Berkeley et al., 1997; Roth et al., 2001]. However, a growing number of institutions are conditioning rhinoceroses to allow frequent blood collection, and thus more serum hormone data will likely become available in the near future.

Comparisons between ultrasound data from this successful pregnancy and those obtained from the five previous pregnancies in this rhinoceros (all of which were lost within the first 90 days) did not reveal any obvious disparities. The size of the preovulatory follicles, timing of ovulation postmating, size of the embryonic vesicle when first observed, and early embryonic growth all were comparable to what had been reported previously for the failed pregnancies [Roth et al., 2001]. Interestingly, this pregnancy was the rhino's second resulting from a cycle in which both ovaries developed preovulatory follicles and ovulated. In the previous case, two embryonic vesicles formed, but both were lost within 30 days [Roth et al., 2001]. In the present case, only one of the two vesicles became atretic, while the second vesicle continued to develop and expand in size.

Similarities between ultrasound images of early rhino embryos and those of horses have been reported previously [Radcliffe et al., 1997, 2001; Roth et al., 2001], and the current data further support those observations. The growth rate curve of the Sumatran rhino embryonic vesicle is very similar in shape to that of the embryonic vesicle of the domestic horse [Ginther, 1995], with two fairly linear rapid expansion phases broken up by a plateau phase during which the vesicle adheres to the uterus and the embryo proper first forms. Although both species demonstrate a similar pattern in that regard, they differ in embryonic vesicle growth rates. For example, the rhino embryo is first visible as a 5-mm-diameter vesicle at 14 days, whereas a day-14 horse embryonic vesicle is already 14 mm in diameter. Some of this difference can be explained by the way data were recorded for the two species. In the horse, day 0 represents the day of ovulation, whereas in this study day 0 was the day of mating. Since ovulation typically occurs within 46 hr of mating [Roth et al., 2001], it might be more appropriate the embryonic vesicles of a day-12 horse with those of a day-14 rhino. In that case, the difference in size would be reduced (day-12 horse embryo = 8 mm). However, during the second growth phase, the rhino embryonic vesicle quickly surpasses that of the horse: it reaches 70 mm in diameter by day 40, when comparably aged horse embryonic vesicles measure only 48 mm (44 mm for day-38 embryos [Ginther, 1995]).

Rectal examinations using a standard 5.0-MHz linear probe were very effective in monitoring the progress of the pregnancy and fetal growth and viability for the first 80 days of pregnancy. In addition to obtaining measurements of embryonic vesicle size and fetal CRL, we noted that the placenta invaded the nongravid horn, which filled with allantoic fluid during days 52–63. This event also occurs in the horse at a similar stage of pregnancy (days 48–57) [Ginther, 1995]. An image of the fetus on day 73 enabled us to determine its sex by identifying the location of the genital tubercle. This method is used to sex horse [Ginther, 1995] and black rhino [Radcliffe et al., 2001] fetuses at a similar age.

Ultrasound monitoring later in gestation proved challenging. After day 80, the fetus descended over the pelvic brim, which made it impossible to perform rectal imaging using current technologies. Transabdominal scanning through the thinner mammary gland tissue with a 3.5-MHz convex probe often provided images of the fetus until mid gestation. After 8 months, the fetus shifted cranially and was rarely seen until late gestation. Some of the difficulties we encountered in imaging the fetus may be overcome with more technical experience in monitoring pregnancy in this species. However, the challenges appear largely due to the anatomy of this rhino species and the associated positioning of the fetus during later pregnancy. Radcliffe et al. [2001] reported that in the black rhinoceros, the fetus is usually visible during rectal ultrasound examinations throughout pregnancy. However, they also reported some difficulties at 6–9 months of gestation.

Because altrenogest supplementation does not alter serum progesterone levels [Squires et al., 1983; Jackson et al., 1986], we were able to evaluate natural endogenous progesterone production throughout gestation. Progesterone concentrations during early pregnancy were similar to those reported previously for three pregnancies that were lost within the first 3 months [Roth et al., 2001]. Therefore, if luteal insufficiency was the cause of embryo loss, it is likely this pregnancy would also have failed. Since we have no data on serum progesterone concentrations during a successful pregnancy in a nonsupplemented Sumatran rhinoceros, it is impossible to draw any conclusions regarding luteal insufficiency as the cause of the previous failed pregnancies. However, the female rhinoceros was young and healthy, and had never exhibited any signs of endometritis, the most common cause of early pregnancy loss. Therefore, she was treated for undiagnosed luteal insufficiency, and the first pregnancy during which she received the altrenogest supplement was successful.

There was a distinct decrease in progesterone during an approximately 4-week period (days 120–150). These concentrations were still above luteal levels, and the decrease occurred later in gestation than all of the previous miscarriages. Therefore, it is unlikely that this shift in hormone levels is linked to the female's history of abortion. In fact, this dip in progesterone was similar to that which occurs in the horse on days 120–150 of gestation and is associated with a transition from ovarian progesterone to placental progestagen production [Holtan et al., 1979]. It has been well documented that progesterone metabolite concentrations in African and Indian rhino fecal samples rise above luteal levels approximately 3 months after conception [Kasman et al., 1986; Hodges and Green, 1989; Schwarzenberger et al., 1993, 1998, 2000; Berkeley et al., 1997; Radcliffe et al., 2001; Patton et al., 1999; Brown et al., 2001], and these data support the hypothesis that a transition in progesterone source, similar to that in the horse, occurs in the rhinoceros. Although the antibody utilized in this study was developed against 4-pregnen-11-ol-3,20-dione hemisuccinate: BSA progesterone, it cross-reacts to some degree with several progestagens [Munro and Stabenfeldt, 1984]. Therefore, the measurable rise in progesterone after day 150 may reflect an increased detection of progestagens even as progesterone declined. In African rhinoceroses, progesterone metabolite concentrations appear to decline a week or two prior to parturition [Berkeley et al., 1997; Radcliffe et al., 2001; Brown et al., 2001]. Although the Sumatran rhino's serum progesterone concentration was at peak levels (15.89 ng/ml) just 9 days before parturition, it did appear to decrease during the final week,

dropping to 6.35 ng/ml just 72 hr before parturition and reaching baseline 24 hr after the calf's birth.

Unfortunately, our attempts to measure serum estrogen concentrations did not provide any useful information. Although it has been reported that urine estrogen metabolite monitoring can be used to track the Sumatran rhino estrous cycle [Heistermann et al., 1998], our efforts to detect significant increases in serum estrogen concentrations that correlated with the day of mating failed (unpublished data). Therefore, it was not surprising that efforts to detect patterns in estrogen production using pooled pregnant sera were uninformative. Although it is possible that the primary form of estrogen in Sumatran rhino serum is unusual and is not recognized by the same antibodies that have recognized estrogen in a broad range of other species, it is more likely that Sumatran rhinoceroses simply produce very low levels of estrogen, and our assays were not sensitive enough to detect dynamic patterns at these low concentrations. In the case of the Sumatran rhinoceros, inherently low estrogen concentrations may, in part, explain the lack of estrous behavior exhibited by the female rhinoceros. In contrast, significant increases in estrogen can easily be detected in urine and feces collected from the Indian rhinoceros [Kassam and Lasley, 1981; Roth and Brown, 1999; Schwarzenberger et al., 2000], and this species displays very strong estrous behavior correlated with preovulatory estrogen peaks. Another potential explanation for our difficulties in detecting serum estrogen is that estrogen production in the Sumatran rhinoceros does not increase during gestation as it does in the horse [Ginther, 1995]. Estrogen metabolite data from the Indian and African black rhinoceroses indicate that estrogen concentrations remain low and unchanging throughout gestation [Schwarzenberger et al., 2000; Brown et al., 2001], and this pattern may hold true for all rhino species.

Because of the difficulties we encountered in imaging the fetus during the second half of pregnancy, and our inability to measure serum estrogen concentrations (a useful indicator of fetal health in horses [Pashen and Allen, 1979]), we employed less direct means of assessing fetal viability. Since we were able to monitor serum progesterone concentrations, the sustained elevation of this hormone and its continued rise throughout pregnancy provided some reassurance that the pregnancy was progressing well. Additionally, allantoic fluid quality was examined during each rectal ultrasound examination. Because an increase in echogenic, floccular material typically occurs following fetal death, due to the sloughing of cells, the image of clear, dark allantoic fluid was another sign that the fetus was healthy. Nevertheless, a more direct means of assessing fetal viability would be valuable.

The protein hormone, relaxin, is considered one of the best indicators of pregnancy in the cat because it is produced by the fetoplacental unit [Stewart and Stabenfeldt, 1985; Tsutsui and Stabenfeldt, 1993]. Although the relaxin profile for the Sumatran rhinoceros differs from that of the cat and horse, it might be useful in monitoring pregnancy because it increases from baseline some time after day 200 and before day 320. This is approximately the same stage of gestation when this fetus shifted in position and could no longer be examined by ultrasound. In the present study, the initial increase in relaxin above baseline occurred much later in gestation than it does in the horse [Stewart et al., 1992]. Although it was detectable, this initial increase to 2–5 ng/ml is minor compared to that observed in the horse (>60 ng/ml [Stewart et al., 1992]). In contrast to the horse, and with more similarity to the pig

and rat [Sherwood et al., 1975, 1980], the rhinoceros exhibited a sharp increase in relaxin that coincided with relaxation of the vulva just prior to parturition. Interestingly, the Sumatran rhino's prepartum concentrations of relaxin were higher than those reported for any other species, even though the antibody used in the assay was made against porcine relaxin. From an applied perspective, if this sharp increase in relaxin 2 weeks before parturition is a consistent pattern among pregnant rhinoceroses, it could serve as a useful indicator of pending parturition. However, many more rhinoceroses would have to be monitored before enough data could be collected to confirm this hypothesis.

Prolactin appears to be another hormone that may be a useful indicator of pending parturition. Prolactin levels remained at baseline throughout gestation until just 2 weeks prior to parturition. The increase in prolactin coincided with the first notable mammary gland development. In contrast, many other species exhibit a gradual increase in prolactin throughout gestation [Concannon et al., 1978] or during the second half of gestation [Banks et al., 1983] before they exhibit a prepartum surge.

We based our decision regarding when to cease altrenogest supplementation on two known facts. First, gestation in three other rhino species is 14–17 months, so we anticipated that gestation in the Sumatran rhino would be similar. Second, Regu-Mate® supplementation does not prevent parturition in horses (personal communications from local equine veterinarians). Therefore, the Sumatran rhinoceros was maintained on the full dosage of altrenogest until day 450 of gestation and was then gradually weaned from the supplement. Because the female was no longer receiving the supplement by day 465 and did not initiate labor until day 475, it can be concluded that Regu-Mate® withdrawal did not induce parturition.

Even if we had lacked the technical skills to measure various hormone levels, close observation of this female rhinoceros revealed clear signals of impending parturition. Mammary gland development, teat enlargement, and vulvar relaxation all became evident 2 weeks prior to parturition and coincided with the prepartum increases in relaxin and prolactin. Because of the detailed behavioral observations that were conducted weeks in advance of parturition, the female's behavioral patterns were well known, and the day before parturition, changes in her behavior signaled that she was about to give birth. These changes included several behaviors that predictably were exhibited at a greater frequency, including vocalizing, horn-rubbing, and locomoting. Perhaps most interesting was the change in urination style and frequency. During the 6 weeks of observation prior to labor, the female rhinoceros reportedly squirted urine only a couple of times, and usually urinated in a downward style three or four times in each 12-hr period. However, during the 12 hr period before she gave birth, the female rhinoceros sprayed urine 69 times, and she continued to spray urine frequently after the calf was born and even after he was weaned. It is possible that this transition in urination style is an instinctive behavioral mechanism in primiparous cows that helps protect the calf from potential predators by masking its odor. In studies of wild rhinoceroses, most of the data on cows comes from females with calves, and these animals spray urine 95% of the time [Van Strien, 1985]. One expected behavioral change that did not occur was a decrease in eating. Although female animals frequently lose interest in food near the time of parturition, inappetence was not one of the indicators in this rhinoceros. She ate most of her

food overnight, as well as fresh browse that was placed in her stall just 4 hr before delivery.

The delivery of a healthy, full-term calf by a Sumatran rhinoceros with a history of miscarriages provides yet another case in which altrenogest supplementation is associated with overcoming recurrent pregnancy loss in an exotic species. To determine whether the hormonal data reported for this pregnancy are representative of those for the species, more pregnancies in additional female Sumatran rhinoceroses must be studied. However, these data represent an important first step in elucidating the physiology of pregnancy in the Sumatran rhinoceros. With the success of this study and the detailed data collected throughout, it now is known that the Sumatran rhinoceros has an approximately 16-month gestation (475 days in this instance), which is similar to that in other rhino species. Furthermore, it is evident that although there are many similarities, pregnancy in the Sumatran rhinoceros differs substantially from that in the horse. Most importantly, this work resulted in the successful birth of the first Sumatran rhinoceros bred in captivity in 112 years, an accomplishment that many had begun to believe was impossible.

CONCLUSIONS

1) A Sumatran rhino with a history of early pregnancy loss successfully carried a pregnancy to term while receiving an oral progestin supplement.

2) The Sumatran rhinoceros has a 16-month gestation.

3) The growth curve of the embryonic vesicle in the Sumatran rhino is similar to that in the horse.

4) Serum progesterone concentrations generally increase throughout gestation.

5) Serum relaxin levels increase above baseline midway through pregnancy and then spike 2 weeks prior to parturition.

6) Serum prolactin increases to very high concentrations 2 weeks prior to parturition.

7) Serum estrogen concentrations are undetectable in commonly used estrogen EIA assays.

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